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Coutinho Silva, Luciano; Paiva, Renato; Peixoto Vargas, Daiane; Pedrosa Corrêa da Silva, Diogo;
Barbosa, Sandro; Cravo Herrera, Raírys
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Decontaminant solution on *in vitro* growth of *Byrsonima intermedia* seedlings

Solução desinfestante no crescimento *in vitro* de plântulas de *Byrsonima intermedia*

Luciano Coutinho Silva^I Renato Paiva^{II} Daiane Peixoto Vargas^{III} Diogo Pedrosa Corrêa da Silva^{II}
Sandro Barbosa^{IV} Raírys Cravo Herrera^V

ABSTRACT

Byrsonima intermedia A. Juss., is a medicinal and fruit plant of the Cerrado in which the conventional propagation is difficult due to the presence of extremely lignified endocarps. Although sodium hypochlorite (NaOCl) is widely used in the surface decontamination, there are few reports of its effects on explant growth. The aim of this work was to study the effect of different pH and exposure periods of *B. intermedia* seeds to a NaOCl solution. Seeds were subjected to different exposure periods (1, 5 and 10 minutes) to a NaOCl solution at different pH (5, 7, 10 and 12) and after treatment with NaOCl, embryos were inoculated in a WPM medium with 50% concentration of salts without sucrose, 0.5% agar and pH 5.8 and after 75 days of culture the growth of seedlings was evaluated. The use of NaOCl is effective in the decontamination of *B. intermedia* seeds, independent of pH variation and exposure periods and the parameters such as percentage of normal seedlings, shoot length, and number of leaves are positively affected by the use of NaOCl solution at pH 8.5-8.9 and by increasing the exposure period, however, the number of roots is affected only by increasing the exposure period in the NaOCl solution.

Key words: micropropagation, native plant, tissue culture, pH, NaOCl.

RESUMO

Byrsonima intermedia A. Juss. é uma planta medicinal e frutífera do Cerrado, cuja propagação convencional é difícil, devido à presença do endocarpo extremamente lignificado. Embora o hipoclorito de sódio (NaOCl) seja amplamente utilizado para a desinfestação superficial, existem poucos relatos de seus efeitos sobre o crescimento do explante. Neste trabalho, o objetivo

foi estudar o efeito de diferentes pHs e períodos de exposição de sementes de *B. intermedia* em uma solução de NaOCl. As sementes foram submetidas a diferentes tempos de exposição (1, 5 e 10 minutos) numa solução de NaOCl com diferentes pHs (5, 7, 10 e 12) e, após o tratamento com hipoclorito de sódio, os embriões foram inoculados em meio WPM com a concentração de 50% de sais sem sacarose, ágar a 0,5% e pH 5,8 e, depois de 75 dias de cultivo, o crescimento das plântulas foi avaliado. A utilização de NaOCl é eficaz na desinfestação de sementes de *B. intermedia*, independente da variação do pH ou dos períodos de exposição. A porcentagem de plântulas normais, o comprimento da parte aérea e o número de folhas são positivamente afetados pela utilização de solução de NaOCl a pH de 8,5-8,9 ou pelo aumento do período de exposição, enquanto o número de raízes é afetado apenas pelo aumento do período de exposição na solução NaOCl.

Palavras-chave: micropropagação, planta nativa, cultura de tecidos, pH, NaOCl.

INTRODUCTION

Byrsonima intermedia A. Juss. is a native and medicinal and fruit plant of the Cerrado (HERRERA et al., 2011) that presents difficulties for the propagation by seeds (LORENZI, 2002) due to the presence of extremely lignified endocarps (SOUTO & OLIVEIRA, 2005). As growth rooms and culture media, *in vitro* culture environments present ideal conditions for the growth of fungi and bacteria, thus, the explant decontamination is a critical factor to any

^IDepartamento de Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal da Paraíba (UFPB), Campus I, 58051-900, João Pessoa, PB, Brasil. E-mail: luoutsilva@yahoo.com.br. Correspondent author.

^{II}Setor de Fisiologia Vegetal, Departamento de Biologia, Universidade Federal de Lavras (UFLA), Lavras, MG, Brasil.

^{III}Embrapa Clima Temperado, Pelotas, RS, Brasil.

^{IV}Universidade Federal de Alfenas (UNIFAL), Alfenas, MG, Brasil.

^VUniversidade Federal do Pará (UFPA), Altamira, PA, Brasil.

tissue culture assay that uses plant material from *ex vitro* sources. When this treatment is not successful, the progress of *in vitro* culture studies becomes nonviable (GEORGE et al., 2008).

A decontaminant agent should eliminate microorganisms and, at the same time, not negatively affect the development of the explant and a variety of decontaminants can be used, such as ethanol, hydrogen peroxide, mercuric chloride, silver nitrate and even antibiotics (YILDIZ & ER, 2002; RIBEIRO & TEIXEIRA, 2008). However, sodium hypochlorite (NaOCl) has been widely used in plant surface decontamination, showing high efficiency in combating bacteria and fungi (EMMANUEL et al., 2004), for example: node segments of *Annona* sp (SANTANA et al., 2011); young nodal stems segments of *Maclura tinctoria* L. (GOMES et al., 2010); nodule segments of *Annona glabra* L. (OLIVEIRA et al., 2010); *Rhodiola rosea* L. seeds (TASHEVA & KOSTURKOVA, 2010); flower-stalk sections of *Limonium sinuatum* L. Mill. (LIU et al., 2005); and seeds of *Syngonanthus elegantulus* Ruhland (PÊGO et al., 2013).

The antimicrobial activity of NaOCl is mainly attributed to hypochlorous acid (HOCl) which presents low molecular weight allowing penetration the cell wall of the microorganism (LEN et al., 2002) and its concentration is increased by the addition of NaOCl or by lowering the solution pH. However, HOCl reversibly dissociates into H^+ and OCl^- , which presents less pronounced biocidal action, and the pH of the solution influences the final balance of these elements (CHUN et al., 1997; EMMANUEL et al., 2004).

Although NaOCl is largely used to explants surface decontamination following plant tissue culture experiments, information about the pH of that solution influencing the *in vitro* growth of explants is still scarce. In this context, the aim of this study was to evaluate the growth of *B. intermedia* seedlings cultivated in aseptic conditions after seed decontamination at different periods of exposure to a NaOCl solution with different pH.

MATERIALS AND METHODS

Mature fruits of *B. intermedia* were collected from a natural population in the south of Minas Gerais State, Brazil, located at 918.0m altitude, latitude 21°14'S and longitude 44.9°00'W GRW. After harvesting, fruits were pulped, soaked in sodium hydroxide (NaOH) 0.1M for 5 minutes and then washed in running water for 10 minutes.

The pyrene (endocarp) were dried in the shade and stored at 4°C in paper bags for a week before being manually opened with the aid of a mechanical press allowing the seeds extraction.

Variation of NaOCl solution pH

In this work, commercial NaOCl presenting 2% active chlorine and pH 11.6 was used to seeds surface decontamination. The solution pH was adjusted with HCl and NaOH, both at 0.1N, using the Tec-3MP TECNAL pH meter.

Decontamination and *in vitro* culture

In laminar airflow, the seeds were immersed in alcohol 70% (v/v) for 30 seconds, then in a solution of NaOCl 50% (v/v) with 1.0% active chlorine (final concentration). Four different variations of pH (5, 7, 10 and 12) combined with three exposure times (1, 5 and 10 minutes) were tested performing an experiment of 12 treatments (NaOCl pH x exposure times) with 15 replicates and just after the exposure to the decontaminant solution, the seeds were washed three times in sterile distilled water, and their coats were removed with forceps.

The culture medium used was WPM (LLOYD & McCOWN, 1980), with 50% of the salt concentration, no sucrose, 0.5 % agar and pH adjusted to 5.8 prior to autoclaving at 121°C for 20 minutes according to the protocol established by NOGUEIRA et al. (2004); only one embryo was inoculated in each test tube (25x150mm) containing 10mL of WPM culture medium and each tube represented one replicate.

After inoculation, the embryos were maintained in a growth chamber under irradiance of $63\mu\text{mol m}^{-2}\text{s}^{-1}$, 16-hour photoperiod and temperature of $25\pm 2^\circ\text{C}$. At 75 days of cultivation the following parameters were evaluated: (i) percentage of normal seedlings; and for each explant, (ii) shoot length, (iii) longest root length, (iv) number of leaves and (v) number of roots.

The measurement of shoot length and longest root was performed using a calliper and for the number of leaves, all those below the first apical pair, were counted. For number of roots, the total number was recorded and regarding the development of normal seedlings, it was expected that they should present normal growth of roots and shoots.

Statistical analyses

Prior ANOVA, performed using SISVAR® statistical software (FERREIRA, 2011), the data were transformed through the square root of $Y+0.5$ and the

averages analyzed by linear regression ($P \leq 0.05$) in a completely randomized factorial design: four (pHs) x three (exposure time).

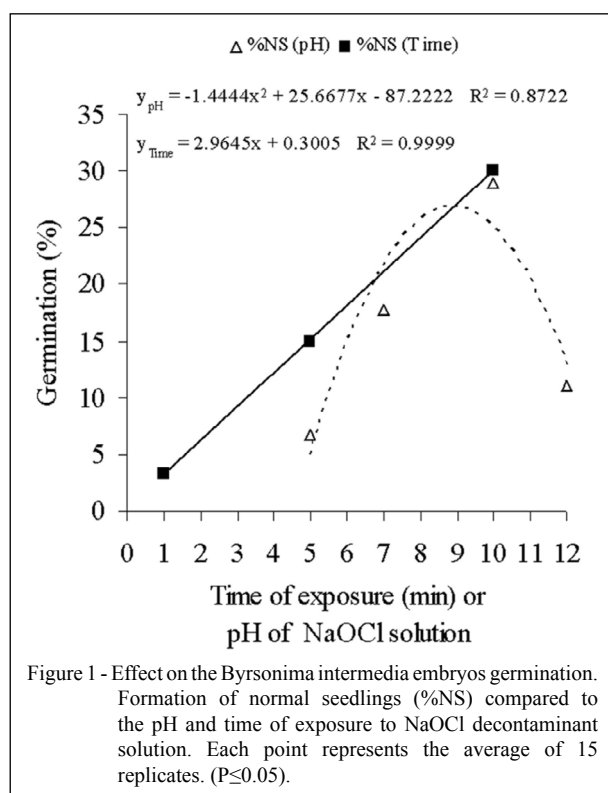
RESULTS AND DISCUSSION

According to ANOVA, significant differences were observed for variation of the pH of NaOCl solution to the parameters: shoot length ($p=0.0232$); number of leaves ($p=0.0183$) and number of roots ($p=0.0436$) per explant; and percentage of normal plants ($p=0.0142$). For the variable period of exposure to NaOCl solution, the parameters: shoot length ($p=0.0148$), longest root length ($p=0.0007$); number of leaves ($p=0.0063$) and number of roots ($p=0.0027$) per explant; and percentage of normal plants ($p=0.0002$) were also significant. The longest root length was not affected by the pH of the NaOCl and the interaction: (pH of NaOCl solution x period of exposure) showed no significance for any parameter assessed. The highest percentage of normal plants (26%) was achieved at pH 8.9, according to derivation of regression equation, or when the seeds were exposed to a NaOCl solution for 10 minute (29.9%) (Figure 1).

According to LORENZI (2002) the *Byrsonima* genus usually presents a low germination

rate and slow seedling emergence. In addition, the embryo extraction process is very harmful and could cause mechanical damage in the embryonic axis, thus reducing the percentage of normal seedlings. Moreover, in the cotyledons, numerous droplets of phenolic compounds are found and the embryonic axis is very short (SOUTO & OLIVEIRA, 2005). Phenolic compounds may prevent germination occurring, and its effects are already well known, for instance, papaya seed germination is affected by the presence of phenols in the sarcotesta (TOKUHISA et al., 2007) and, according to TESIO et al. (2011), shoot extracts from *Helianthus tuberosus* L. contain bioactive phenolic compounds, able to cause inhibitory effects on lettuce seed germination and seedling growth. Thus, physical and/or chemical factors related to *B. intermedia* seeds may have affected the germination leading to this low percentage of normal seedlings.

The best performance for shoot length (2.7mm), number of leaves (1.8) and number of roots (0.7) was achieved with NaOCl solution at pH 8.5, 8.7 and 8.7, respectively, according to the equations for these three parameters (Figure 2a). Regarding the duration of exposure to this solution, we observed an increasing linear effect for shoot length (2.6mm), longest root length (9.9mm), number of roots (0.7) and number of leaves (1.8) when the equation was



applied for 10 minutes of exposure, respectively (Figure 2b).

Regardless of the variation of the pH of NaOCl or the period of exposure to this solution, decontamination was 100% efficient. We conducted an additional treatment (control), which presented 100% contamination, where seeds were only immersed in 70% alcohol (v/v) for 30 seconds and then rinsed in sterile distilled water three times before removing the integuments.

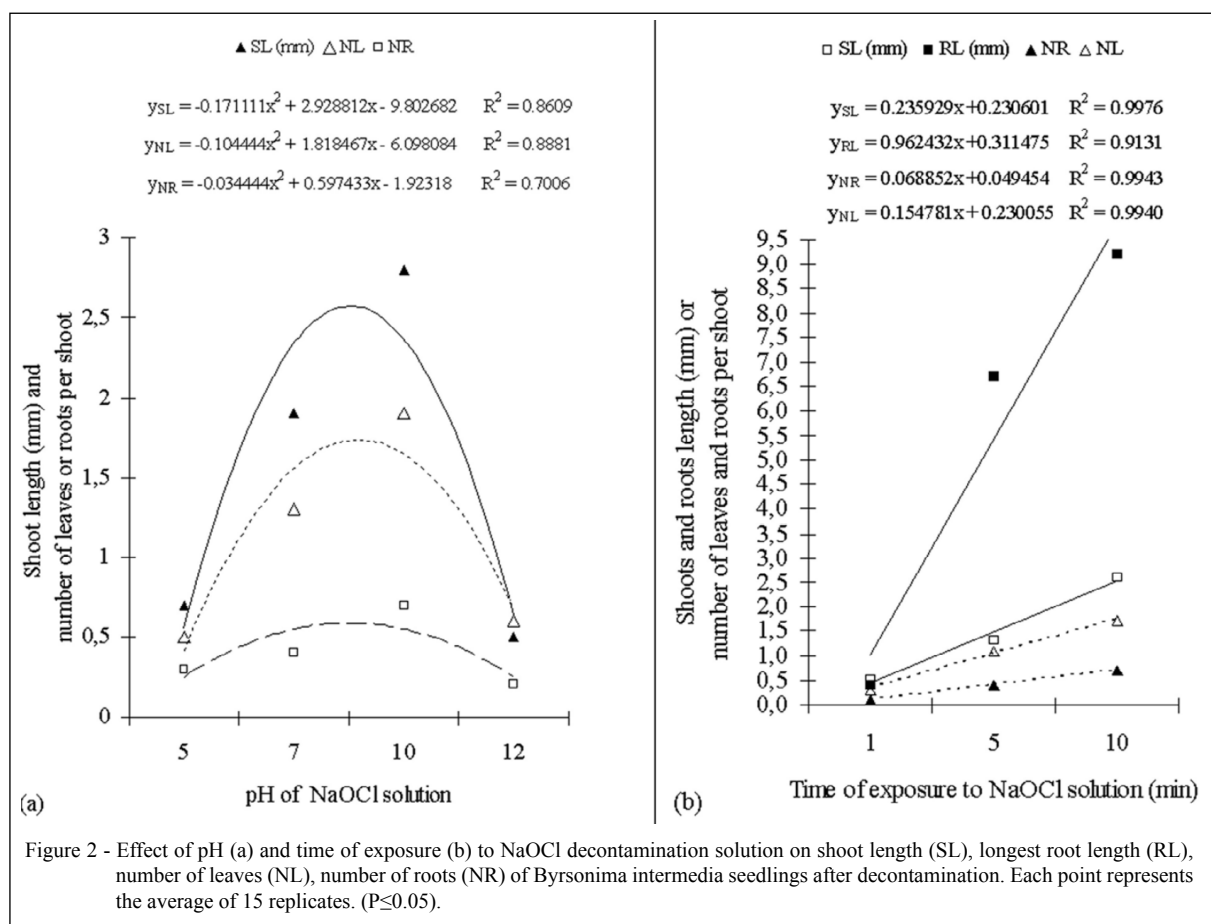
In this work, decontamination was efficient at all pHs tested, which differs from results reported by CHUN et al. (1997) where these authors showed that lowering the pH of NaOCl increased the efficiency of decontamination of rice seeds where, bacterial and fungal contaminations were effectively removed at pH 2-7 and 2-5, respectively, however, the highest germination rate (100%) was achieved when rice seeds were treated with NaOCl at pH 11.6.

Apparently the triple-rinse routinely applied to explants undergoing decontamination does not remove all the components of the NaOCl solution, maintaining a residual effect on the explant.

A reduction of *in vitro* contamination due to residual effects from the chlorinated water used for rinsing of glassware was showed by TEIXEIRA et al. (2008) when these authors inoculated explants of *Eucalyptus pellita* F. Muell in the culture medium without autoclaving, contamination was 100%.

When the tubes were soaked in a NaOCl solution with 0.001% of total active chlorine, with subsequent distribution of non-sterile culture medium in laminar airflow, the contamination was reduced by 60%, thus confirming the effect of residual components in NaOCl. A positive effect on the growth of *in vitro* *Sequoia sempervirens* L. seedlings was reported by (RIBEIRO, et al., 2011) when NaOCl was used as a sterilizing chemical medium.

This residual effect was also observed even in control of endogenous contaminants (TEIXEIRA et al., 2008) and these authors observed that culture media treated with concentrations of less than 0.005% of total active chlorine, showed contamination at the base of the explant, featuring endogenous contamination. When these authors used culture media treated with concentrations greater than



0.005% of total active chlorine, no contamination was found, reaffirming the residual effect of the agent, in this case, acting as a culture medium sterilizer.

Although all decontamination treatments have been effective, seedling growth was positively affected using NaOCl at pHs ranging 8.5-8.9, providing pronounced seedling growth when the seeds were exposed for 10 minutes at that solution. At these pHs, both components HOCl and OCl⁻ are present in the solution in equivalent amounts (EMMANUEL et al., 2004). Thus, triple-rising may not completely remove these components of the NaOCl solution and the residual effect could continue to positively influence the growth of explants. However, the use of NaOCl solution in highly alkaline pH positively affected rice seedling development. But, when the seeds were treated with NaOCl at pH 11.6 was observed 100% germination, compared to 42% when treated at pH 3 (CHUN et al., 1997).

The use of NaOCl in the culture medium as a chemical sterilizer, not only was effective in sterilizing the medium, but also favoured the elongation of shoots of *E. pellita* (TEIXEIRA et al., 2008). The increase in the number and fresh weight of shoots of pineapple was confirmed when NaOCl was added in the culture medium (TEIXEIRA et al., 2006), and this increase could be linked to photosynthesis as chlorine presents a catalytic role in the photosynthetic process, in addition, chlorine is a micro nutrient (EMMANUEL et al., 2004).

In this experiments, was proved that the pH of NaOCl influences seedling development once the largest growth of *Byrsonima intermedia* seedlings can be directly related to the residual effect of NaOCl solution used at different pHs to seeds decontamination which allowed a greater interaction with the explant.

CONCLUSIONS

The use of NaOCl was effective for *Byrsonima intermedia* seeds decontamination independently of the pH variation or period of exposure to that solution; for a better seedling growth an intermediate range of pH 8.7 and 10 minutes of exposure to NaOCl decontamination solution is recommended.

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